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(54) Title: 4-OXO-FENRETINIDE, ADMINISTERED ALONE AND IN COMBINATION WITH FENRETINIDE, AS PREVENTIVE AND THERAPEUTIC AGENT FOR CANCER

(57) Abstract: A drug based on a metabolite of fenretinide, or N-(4hydroxyphenyl)retinamide (4-HPR), specifically 4-oxo-N-(4-hydroxyphenyl)retinamide (4-oxo-4-HPR), is used in the treatment of different kinds of tumors, in particular in the treatment of ovarian carcinoma, breast tumor and neuroblastoma. The drug is more potent than fenretinide alone, and enhances the effects of fenretinide when administered together with fenretinide.



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4-oxo-fenretinide, administered alone and in combination with fenretinide, as preventive and therapeutic agent for cancer.

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Technical Field

The present invention relates to the clinical use of one of the metabolites of the synthetic retinoid fenretinide, which is known to have antitumor properties. In particular it relates to the use of the metabolite 4-oxo-fenretinide for preventing and treating cancer and for its use in combination with fenretinide in order to improve fenretinide activity and/or to overcome fenretinide resistance.

15

Background of the invention

Retinoids are natural and synthetic derivatives of vitamin A (retinol), which modulate different cellular processes, including proliferation, differentiation and apoptosis. They are compounds of clinical interest for the treatment and prevention of a variety of tumors. Fenretinide, or N-(4-hydroxyphenyl)retinamide (4-HPR), is a synthetic retinoid (an amide of all-trans retinoic acid), which, in preclinical models, proved to be less toxic than many other retinoids while maintaining a significant biological activity. (Moon et al., Cancer Res., 1979, 39:1339-1346). In animal models, 4-HPR had preventive efficacy in mammary, prostate and colon carcinogenesis and

lymphomagenesis and therapeutic efficacy against neuroblastoma, mammary, ovary and Kaposi's tumors (Formelli et al., FASEB J. 1996, 10:1014-1024; Ferrari et al., Clin. Cancer Res. 2003, 9:6020-6029). In clinical trials in
5 adults (Veronesi et al., J. Natl. Cancer Inst. 1999, 91:1847-56) and in children (Garaventa et al., Clin. Cancer Res. 2003, 9:2032-2039), 4-HPR had a favorable toxicity profile and was better tolerated than other retinoids. Its main side effect was impaired dark adaptation, which was
10 due to reduction of retinol plasma levels (Formelli et al., Cancer Res. 1989, 49:6149-6152). In humans, 4-HPR has shown efficacy in premalignant lesions such as oral leucoplakia (Chiesa et al., Oral Oncol. Europ. J. Cancer, 1992, 28B(2):97-102), lichen planus (Tradati et al., Cancer
15 Lett., 1994, 76:109-111) and actinic keratoses (Moglia et al., Cancer Lett. 1996, 110:87-91) and has shown promising results for the prevention of breast and ovarian tumors (Veronesi et al., J. Natl. Cancer Inst. 1999, 91:1847-56; De Palo et al., Gynecol. Oncol., 2002, 86:24-27). In a
20 clinical trial in women treated with 4-HPR after having undergone surgery for early breast cancer, 4-HPR reduced the incidence of new breast cancer in premenopausal women (Veronesi et al., J. Natl. Cancer Inst. 1999, 91:1847-56). In the same trial, a significant reduction in the incidence
25 of ovarian cancer was also observed during the treatment period (De Palo et al., Gynecol. Oncol., 2002, 86:24-27). Actually, the retinoid is under investigation as a preventive and therapeutic agent for neuroblastoma, breast

and ovarian tumors.

The tumor preventive and therapeutic activity of 4-HPR has been attributed to its potent growth inhibitory effects, associated with induction of apoptosis, which have
5 been shown in tumor cells of different histiotypes, including breast, prostate, ovary, head and neck, neuroblastoma and leukemia (Formelli et al., FASEB J. 1996, 10:1014-1024). Considerable attention has been directed towards understanding the mechanism of action of this
10 retinoid and different molecular targets have been implicated in its growth inhibitory activity.

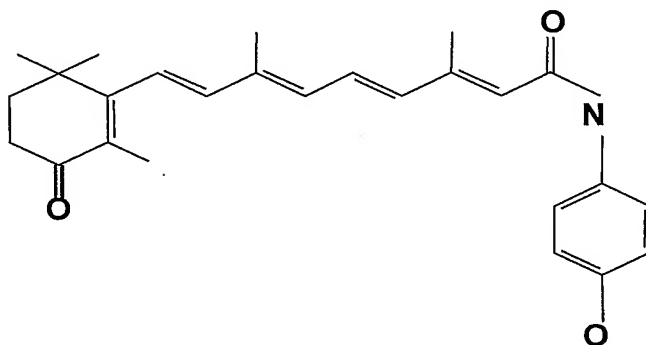
An important aspect that may contribute to the biological effects of 4-HPR is its metabolism. It is known that 4-HPR is extensively metabolized in vivo (Hultin et
15 al., Drug Metab. Dispos., 1986, 14:714-717; Formelli et al., Cancer Res., 1989, 49:6149-6152 ; Mehta et al., Eur. J. Cancer, 1991, 27:138-141), but it is not known whether 4-HPR or one of its metabolites is the active agent and how they interact with each other. Moreover, nothing is known
20 about 4-HPR metabolism in tumor cells. To date, the only identified metabolite of 4-HPR is N-(4-methoxyphenyl)retinamide (4-MPR), which is less polar than the parent drug and which has been found in all the in vivo studies in mice, rats and humans. 4-MPR does not seem to
25 play a role in 4-HPR tumor growth inhibitory effect because it was ineffective in inhibiting proliferation in different tumor cell lines (Appierto et al., Br. J. Cancer., 2001, 84:1528-1534; Metha et al., Eur. J. Cancer, 1998, 34:902-

907). Moreover, in mice bearing a human ovarian carcinoma, 4-MPR had no antitumor effect and it did not increase 4-HPR activity (Formelli et al., Eur. J. Cancer, 2000, 36:2411-2419). In addition to 4-MPR, all the indicated studies also
5 reported the presence of unidentified metabolites more polar than the parent drug. In one of these studies, it was found that in normal and tumor breast tissues of women treated with 4-HPR, 4-MPR was mainly localized in the fat tissue, whereas 4-HPR and an unidentified polar metabolite
10 were concentrated in epithelial cells, that is in the cells where the drug is supposed to exert its effect (Metha et al., Eur. J. Cancer, 1991, 27: 138-141).

Summary of the invention

15 The present invention provides a metabolite of fenretinide, or N-(4-hydroxyphenyl)retinamide (4-HPR), specifically 4-oxo-N-(4-hydroxyphenyl)retinamide (4-oxo-4-HPR) of the following formula:

20



25

Chemical formula: $C_{26}H_{31}NO_3$

Molecular weight: 405

for treatment and prevention of cancer and for improving and/or overcoming fenretinide resistance.

5 4-oxo-4-HPR, is a polar metabolite of 4-HPR, which was identified in tumor cells treated with the retinoid and in plasma samples collected from patients enrolled in 4-HPR clinical trials.

10 The molecule was synthesized and in in vitro grown tumor cells 4-oxo-HPR had a strong antiproliferative activity in 4-HPR-sensitive and -resistant cells, was more potent than 4-HPR, and it was synergic with 4-HPR.

15 Administered to mice, 4-oxo-HPR caused a slight reduction of retinol plasma levels, which was lower than that caused by 4-HPR. It is therefore an object of the present invention to provide 4-oxo-4-HPR which is effective in tumor cells, including those resistant to 4-HPR, and whose effects on retinol are lower than those of 4-HPR, for preventing and treating the development of malignant
20 lesions. In addition, the results of the two retinoids in combination indicate a way to improve 4-HPR activity and/or to overcome 4-HPR resistance.

Brief Description of the Drawings

25 Fig. 1 shows the results of mass spectrometry analysis in positive mode for the identification of the polar metabolite;

Fig 2. reports the average values \pm SD of a series of

experiments concerning the antiproliferative activity of 4-oxo-4-HPR and 4-HPR in ovarian tumor cell lines;

Fig 3. reports the average values \pm SD of a series of experiments concerning the antiproliferative activity of 4-oxo-4-HPR and 4-HPR in breast tumor cell lines;

Fig 4. reports the average values \pm SD of a series of experiments concerning the antiproliferative activity of 4-oxo-4-HPR and 4-HPR in neuroblastoma tumor cell lines;

Fig. 5 reports the results of apoptosis assays, whereby DNA fragmentation in treated cells is expressed as fold versus DNA fragmentation in control cells; and

Fig. 6 reports the results of experiments carried out with the combination of 4-oxo-4-HPR and 4-HPR, showing the antiproliferative effect of the combined treatment.

Detailed Description of the Invention

The present invention provides a drug that may be used for the prevention and treatment of cancer, that is a metabolite of the antitumor agent 4-HPR, specifically 4-oxo-HPR, that is more potent and with a wider range of tumor cell growth inhibitory activity than the parent drug.

The compound may be identified in tumor cells and in blood samples of patients treated with 4-HPR and synthesized according to the following examples.

Identification of 4-oxo-4-HPR

Example 1

We have previously reported that in plasma of patients

treated with 4-HPR (Formelli et al., Cancer Res., 1989, 49:6149-6152) and in A2780/HPR human ovarian carcinoma cells, obtained from A2780 cells after continuous exposure to 5 μ M 4-HPR (Appierto et al., Br. J. Cancer, 2001, 84:1528-1534), besides the parent drug 4-HPR and the metabolite 4-MPR, another metabolite, more polar than the parent drug, was present. Here we describe how the metabolite can be identified.

The presence of 4-HPR and its metabolites in plasma, culture medium and tumor cell extracts was evaluated by high pressure liquid chromatography (HPLC) as previously described (Formelli et al., Cancer Res., 1989, 49:6149-6152). Briefly, plasma samples were obtained from blood collected 12h after the last daily 4-HPR dose from patients participating in a breast cancer prevention trial (Formelli et al. Cancer Res., 1989, 49: 6149-6152). A2780/HPR -cell pellets and -media were collected 72h after treatment with 5 μ M 4-HPR (Appierto et al., Br. J. Cancer, 2001, 84:1528-1534). All the procedures were performed with the samples protected from light. Cell pellets were resuspended in 1 ml of distilled water containing 125 μ g/ml of butylated hydroxytoluene (BHT) (Sigma) as anti-oxidant and sonicated. An aliquot of 200 μ l of each plasma, cell and medium sample was added to 400 μ l CH_3CN containing 125 μ g/ml BHT, and the mixture was vortexed and centrifuged to pellet the precipitated proteins. The recovered supernatants were analyzed on a liquid chromatograph (Perkin Elmer, Norwall, CT) fitted with a C18 (5 μ m) reverse-phase column (150x4.6

mm) and a C18 precolumn (Perkin Elmer, Milan, Italy). The mobile phase consisted of $\text{CH}_3\text{CN}:\text{H}_2\text{O}:\text{CH}_3\text{COOH}$ (75:23:2, vol/vol/vol) delivered at a flow rate of 2 ml/min. Detection was performed with a Perkin Elmer LC95 absorbance
5 detector at 340 nm. The reference standard 4-HPR, was supplied by the R.W. Johnson Pharmaceutical Research Institute (Spring House, PA, USA).

Identification of the polar metabolite was performed by atmospheric pressure chemical ionization mass
10 spectrometry (APCI-MS) analysis in positive mode. APCI mass spectra were acquired using a ThermoQuest LCQDeca mass spectrometer (Finnigan Mat, San Jose, CA, USA) equipped with an atmospheric pressure chemical ion source and an Xcalibur data system and connected to an HPLC system. The
15 conditions of HPLC separation were the same as described above with the following modifications: the flow rate of the mobile phase was 1 ml/min and the size of the C18 column was 125x3mm. Operating parameters of the APCI-MS interface were optimized using flow injection of 4-HPR in
20 the mobile phase utilized for HPLC separation at a flow rate of 10 $\mu\text{l}/\text{min}$. Optimum conditions included: sheath gas flow of 80 arbitrary units, vaporized temperature of 300°C, discharge current of 6 μA , capillary voltage of 3 V, capillary temperature of 250°C, fragmentor voltage (used
25 for collision-induced dissociation) of 30%. Mass spectra were acquired over a range m/z 200-600.

The results of mass spectrometry analysis in positive mode are reported in Fig. 1. The polar metabolite was a

compound with a positive molecular ion $[M+H]^+$ at m/z 406. Fragmentation of the positive molecular ion with m/z 406 yielded, by loss of the amino-phenyl-hydroxy group, the fragment ion $[M+H]^+-(NH_2-C_6H_4-OH)$ at m/z 297, having 14 units
5 over the fragment ion recorded in ms^2 of 4-HPR. The following fragmentation of this ion made it possible to record a ms^3 spectrum containing a fragmentation pattern in which the main fragments differed again by 14 units from those present in the pattern recorded in the ms^3 spectrum
10 of 4-HPR, suggesting that 4-HPR was oxidized in the retinoic ring. The compound, by ms^2 and ms^3 , was therefore characterized to be the 4-oxo-4-HPR.

Synthesis of 4-oxo-4-HPR

15

Example 2

For the synthesis of 4-oxo-4-HPR, methyl 4-oxoretinoate was prepared as previously described (Curley and Carson, Drug Des. Del., 1987, 1:219-224) with slight modifications. That is, the unstable methyl 4-
20 bromoretinoate was prepared as described and then treated with 9:1 acetone/water containing 1.5 equivalents of potassium carbonate, instead of the previously used potassium acetate, in order to generate methyl 4-hydroxyretinoate directly. The latter compound was then
25 converted to 4-oxoretinoic acid as previously described. The 4-oxoretinoic acid (13 mg) was activated as its acid chloride and treated with 4-aminophenol using the method of Villeneuve and Chan Tet. Lett., 1997, 38:6489-6492.

Purification of the resultant 4-oxo-4-HPR by silica gel preparative thin-layer chromatography (1:1 ethyl acetate/hexane) gave a 28% yield (4.6 mg) of product as a yellow oil with the following properties: UV (methanol) λ_{max} 371 nm (ϵ 63,210); ^1H NMR (acetone- d_6) δ 1.26 (s, 6, $(\text{CH}_3)_2$), 1.82 (s, 3, 5- CH_3), 1.84 (t, 2, 2- CH_2), 6.01 (s, 1, 13-H), 6.35-6.42 (m, 4, vinylns), 6.74 (d, 2, Ar, $J = 8.9\text{Hz}$), 7.06 (dd, 1, 11-H), 7.52 (d, 2, Ar, $J = 8.9\text{ Hz}$), 8.12 (s, 1, O/N-H), 9.03 (s, 1, O/N-H); HPLC on a Beckman Instruments (San Ramon, CA, USA) model 127 pump with model 166 detector, using a 250x4.6 mm Ultrasphere ODS column with 85% methanol/water at 1 ml/min, $t_R = 4.8$ min (>95% purity); MS (electrospray) for $\text{C}_{26}\text{H}_{31}\text{NO}_3 + \text{Na}$, calculated 428.2202, observed 428.2198. The UV spectrum was recorded on a Beckman Instruments DU-40 while ^1H NMR spectra were recorded at 400 MHz on a Bruker Instruments (Billerica, MA, USA) spectrometer. The MS on the synthetic material was recorded on a Micromass (Milford, MA, USA) QTOF Electrospray mass spectrometer.

20

Biological effects of 4-oxo-4HPR

Growth Inhibition of tumor cells

The antiproliferative activity of 4-oxo-4-HPR in human ovarian, breast, and neuroblastoma tumor cell lines was determined and compared with that of 4-HPR.

The following human ovarian carcinoma cell lines were tested: A2780 was obtained from Dr. R.F. Ozols (National

Cancer Institute, Bethesda, MD, USA); IGROV-1 was obtained from Dr. J. Benard (Villejuif, France); OVCA432 was obtained from Dr. R.C. Knapp (Boston, MA, USA); OAW42 and SKOV-3 were purchased from ATCC (Rockville, MD, USA).

5 A2780/HPR, a 4-HPR-resistant ovarian carcinoma cell line, was obtained in our laboratory by continuous exposure of A2780 cells to increasing concentrations of 4-HPR as previously described (Appierto et al., Br. J. Cancer, 2001, 84:1528-1534; Prinetti et al., J. Biol. Chem., 2003, 10 278:5574-5583). Briefly, cells surviving 60 transplants in 4-HPR -containing medium (3 transplants at 1 μ M, 11 transplants at 2 μ M, 8 transplants at 3 μ M, and 38 transplants at 5 μ M) were cloned by limiting dilution. One clone, A2780/HPR, was expanded and, when tested for 4-HPR 15 sensitivity, demonstrated a 10-fold resistance to 4-HPR, which was slightly reversible upon drug removal for five transplants. Therefore, A2780/HPR cells were continuously maintained in 5 μ M 4-HPR and seeded without 4-HPR in all the experiments performed to test their sensitivity to 4-oxo-4-HPR. 20

The following human breast tumor cell lines, obtained from Dr. R.Sutherland (Garvan Institute, Sydney, Australia) were tested: T-47D, BT-20 and MCF-7. The following human neuroblastoma cell lines, purchased from ATCC, were tested: 25 SK-N-BE, SK-N-SH, SK-N-MC, and GI-LI-N. All cell lines were grown in monolayer, in medium containing 10% fetal bovine serum (Invitrogen) in 5% CO₂ at 37 °C. The medium for A2780, A2780/HPR, IGROV-1, OVCA432, SKOV-3, T-47D, BT-20,

MCF-7, GI-LI-N and SK-N-BE cells was RPMI 1640 (Cambrex, Verviers, Belgium) and the medium for OAW42, SK-N-MC and SK-N-SH cells was EMEM (ATCC).

For the growth inhibition assays, cells were seeded at a density of 3500 cells per well in 96 cluster tissue culture plates, and treated 24 hr after seeding with different concentrations of 4-oxo-4-HPR or 4-HPR. 4-oxo-4-HPR was dissolved in absolute ethanol and 4-HPR (obtained from Dr. J. A. Crowell -NCI Bethesda, MD, USA) was dissolved in Me₂SO. 4-oxo-4-HPR and 4-HPR were dissolved at a concentration of 10 mM and stored at -80° under N₂ in the dark prior to further dilution in culture medium at final concentrations ranging from 0.3 to 10 µM, so that the ethanol and Me₂SO concentrations did not exceed 0.1%. Control cultures received the same amount of ethanol or Me₂SO as the treated cultures. 72 hr after treatment cell number was estimated by using the sulforhodamine B assay according to Monks et al., (J. Natl. Cancer, 1991, 11: 757-766). The survival of treated cells was calculated for the various drug concentrations as percentage of the survival of control cells. The antiproliferative activity of 4-oxo-4-HPR and 4-HPR in each cell line, was tested in three separate experiments and the average values ± SD are reported in Figures 2, 3, and 4.

The survival of 5 ovarian tumor cell lines after treatment with various concentrations of 4-oxo-4-HPR or 4-HPR for 72 hr is reported in Figure 2. 4-oxo-4-HPR was more effective than 4-HPR in reducing tumor cell survival in all

tested cell lines. 4-oxo-4-HPR was also highly effective in A2780/HPR cells with induced resistance to 4-HPR, thus showing no cross resistance with the parent drug 4-HPR. Tested in breast tumor cell lines (Figure 3), 4-oxo-4-HPR
5 resulted more potent than 4-HPR in BT-20 and in MCF-7 and similarly effective in T-47D. Among the 4 tested neuroblastoma cell lines (Figure 4), SK-N-BE and SK-N-SH were more sensitive to 4-oxo-HPR than to 4-HPR, SK-N-MC was more sensitive only at the lowest concentration tested (0.3
10 μ M) and GI-LI-N was equally sensitive. The 50% inhibitory concentrations (IC_{50}) of 4-oxo-4-HPR and 4-HPR, calculated from the graphs reported in figures 2, 3, and 4, are reported in Table 1.

The IC_{50} of 4-oxo-4-HPR was lower than that of 4-HPR in
15 all the tested cell lines.

Apoptosis induction

Although the mechanism of action of 4-HPR is not well understood, its tumor growth inhibitory effects have been
20 attributed to apoptosis induction (Supino et al., Int. J. Cancer, 1996, 65:491-497). The apoptosis inducing effects of 4-oxo-4-HPR were compared with those of 4-HPR in the human ovarian carcinoma cell line A2780 and in its counterpart A2780/HPR, with induced resistance to 4-HPR.
25 Apoptosis was evaluated by a Cell Death Detection ELISA Plus Assay Kit (Roche Diagnostic GmbH, Germany) according to the manufacturer's instructions. This assay quantitates the amount of cytoplasmic histone-associated DNA fragments

which increases during apoptosis. Cells were seeded in 96-well cell culture plates and treated the day after with 5 μ M 4-oxo-4-HPR or 4-HPR for 24 hr. DNA fragmentation in treated cells, expressed as fold versus DNA fragmentation in control cells, is reported in Figure 5. In A2780 cells, the induction of apoptosis by 4-oxo-4-HPR was higher than that of 4-HPR. 4-oxo-4-HPR also induced apoptosis in A2780/HPR cells, whereas 4-HPR had no effect.

Antiproliferative effect of the combined treatment of 4-oxo-4-HPR with 4-HPR

Studies were performed to assess whether 4-oxo-4-HPR potentiates the tumor growth inhibitory effects of 4-HPR. A2780 cells were exposed to a range of concentrations of 4-HPR and 4-oxo-4-HPR as single agents and of both agents together at 3 different ratios, namely 1:0.3, 1:1 and 1:3. The fractional survival (f) after 3 days of treatment was calculated for each experimental condition. CalcunSyn software, based on the median-effect analysis method according to Chou and Talalay, as described in Adv. Enzyme Regul., 1984, 22:27-55, was then used to determine the effects of the combination of the 2 drugs. For each level of fractional survival ($f = 0.80, 0.70, . . . , 0.20, 0, 10$), a combination index (CI) was calculated according to the equation:

$$CI = (D)_1 / (D_f)_1 + (D)_2 / (D_f)_2 + \alpha [(D)_1 (D_f)_2 / (D_f)_1 (D_f)_2]$$

where $(D)_1$ and $(D)_2$ are the concentrations of the combination required to produce fractional survival f ,

(D_f)₁ and (D_f)₂ are the concentrations of the individual drugs required to produce f , and $\alpha = 0$ or 1 if the drugs' mechanisms of action are mutually exclusive or mutually nonexclusive, respectively. $CI < 1.0$ indicates synergy, $CI = 1.0$ indicates additivity, and $CI > 1.0$ indicates antagonism. The results of the experiments with the combination of 4-oxo-4-HPR and 4-HPR are presented in Figure 6. At the 3 ratios tested, the combination of the two drugs resulted in a greater degree of growth inhibition compared with those obtained with the two agents alone. Using mutually exclusive assumptions, the CI values were lower than 1 at all concentrations, suggesting a synergistic interaction of the two drugs.

The antiproliferative effect of the combination of the two drugs, administered at one single dose, was also tested in other ovarian carcinoma cell lines and in breast tumor cell lines. The type of interaction between 4-HPR and 4-oxo-4-HPR was assessed by the method of Drevinko et al., as described in Cancer Biochem. Biophys., 1976, 1:187-195. For this evaluation the drugs were assumed to provide independent effects. The combination index (CI) was calculated as follows: survival fraction (SF) of the combination of 4-HPR + 4-oxo-4-HPR divided by (SF 4-HPR x SF 4-oxo-4-HPR). The results for CI are defined as follows: $CI = 1$ indicates an additive effect, $CI < 1$ indicates a supra-additive effect and $CI > 1$ indicates a sub-additive effect. The results of the single experiments are reported in Table 2. When doses of 4-HPR and 4-oxo-4-

HPR which produced almost no growth inhibitory effects were combined, they resulted in a supra-additive effect in all but one cell lines, thus confirming that 4-oxo-4-HPR is able to potentiate 4-HPR tumor growth inhibitory effects.

5

Effects on plasma retinol

One of the main side effects of 4-HPR, is diminished dark adaptation or nyctalopia (Formelli et al., FASEB J., 1996, 10:1014-1024) which is caused by a decrease in plasma retinol levels (Formelli et al., Cancer Res., 49:6149-6152). The influence of 4-oxo-4-HPR on plasma retinol levels was assessed in mice and was compared with the effects caused by 4-HPR. Animal studies were approved by the Ethics Committee for Animal Experimentation of Istituto Nazionale Tumori (Milan Italy) and were carried out according to national and international guidelines (Italian Governing Law, Legislative decree 116, January 27, 1992 and UKCCCR guidelines for the welfare of animals in experimental neoplasia. Br. J. Cancer 1988, 58:109-113).

Female Swiss nude mice (7-9 weeks old) were supplied by Charles River (Calco, Italy) and kept in laminar air-flow rooms in sterilised cages, with bedding, food and acidified water. 4-HPR was obtained from Dr. J. A. Crowell -NCI Bethesda, MD, USA. 4-oxo-4-HPR and 4-HPR were dissolved in absolute ethanol and then diluted in a sterile 0.9% (w/v) NaCl solution containing 1,65 mg/ml bovine serum albumin (Sigma, St Louis, MO, USA) at a final ethanol concentration of 10% (v/v). The drug solutions were freshly prepared

once a week, protected from light and kept at 4°C. Mice were treated i.p. with 120 mg/kg of 4-oxo-4-HPR or 4-HPR for 4 days. The two drugs were delivered at a volume of 10ml/kg of body weight. Control mice were treated with the same solvent of 4-oxo-4HPR and 4-HPR. Three mice per group were used. Blood samples, for drug and retinol measurements, were drawn from the retroorbital plexus of anesthetized mice before and 5 hr after the last treatment, collected into heparinized tubes and wrapped in aluminium foil in order to protect them from light. Retinol and drug concentrations in plasma were assayed by HPLC as previously described (Formelli et al., Cancer Res. 1989, 49:6149-6152) and as above reported (see Exemple 1) and are reported in Table 3. 4-HPR caused a remarkable (56%) reduction of plasma retinol levels, whereas 4-oxo-4-HPR was less potent and caused a reduction only slightly higher than observed in control mice.

The average plasma drug concentrations, found after administration of equal doses, were similar for the two drugs (4-oxo-4-HPR = 783 ng/ml and 4-HPR = 847 ng/ml). In mice treated with 4-HPR the drug was metabolized to 4-oxo-4-HPR, as observed in humans (see Exemple 1).

Table 1

Tumor growth inhibitory effects		
50% Inhibitory Concentrations (IC ₅₀) at 72 h		
Tumor cell lines	4-HPR (μ M)	4-oxo-4-HPR (μ M)
OVARIAN		
A2780	1.5	0.6
A2780/HPR	8	0.5
OVCA432	8	2.5
IGROV-1	5	2
OAW42	10	3
SKOV-3	8	2
BREAST		
BT-20	7	2
T-47D	4	2.5
MCF-7	3	1.5
NEUROBLASTOMA		
SK-N-BE	5	1
GI-LI-N	0.7	0.6
SK-N-SH	5	3
SK-N-MC	0.7	0.4

Table 2

Antiproliferative effect of 4-HPR and 4-oxo-4-HPR as single agents and in combination at 72 h						
	Survival (% control cells)					
	4-HPR		4-oxo-4-HPR		4-HPR+4-oxo-4-HPR	CI ^a
Tumor cell lines	(1 μM)	(5 μM)	(0.3μM)	(1 μM)		
OVARIAN						
OVCA432	115 105			98 102	35 53	0.36 0.53
IGROV-1	97 91			96 76	17 43	0.18 0.30
SW626	96 98			90 98	55 62	0.64 0.70
A2780/HPR		81 89 81	96 84 71		55 35 28	0.70 0.47 0.49
BREAST						
T-47D	90			94	61	0.71
BT-20	89			70	57	0.91
MCF-7	86			56	60	1.25

a) CI = Combination Index calculated as follows: Survival Fraction (SF) 4-HPR + 4-oxo-4-HPR / (SF 4-HPR x SF 4-oxo-4-HPR). CI =1 additive effect; CI < 1 supra-additive or potentiation effect; CI > 1 subadditive effect.

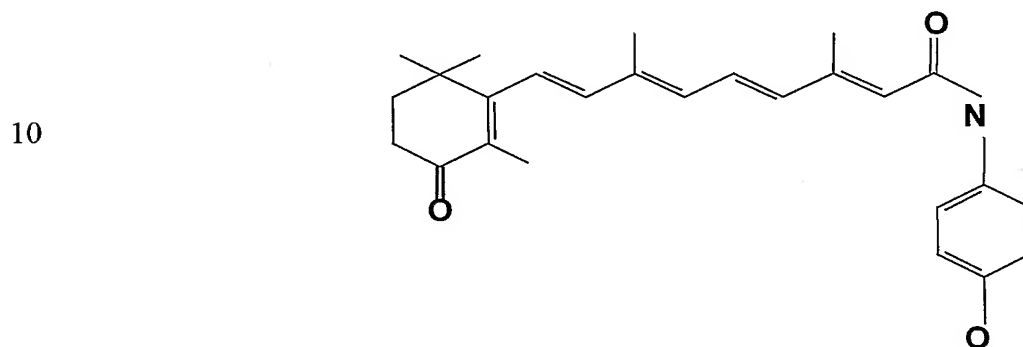
Table 3

Drug levels and retinol level reduction in mice treated with 4-oxo-4-HPR and 4-HPR^a					
Group of treatment	4-HPR (ng/ml)	4-oxo-4-HPR (ng/ml)	Retinol		
			at baseline (ng/ml)	after treatment (ng/ml)	% reduction
Control	0	0	166±39	129±30	22
4-oxo-4-HPR	0	783±117	176±11	107±24	38
4-HPR	847±117	355± 57	156±59	55±20	56

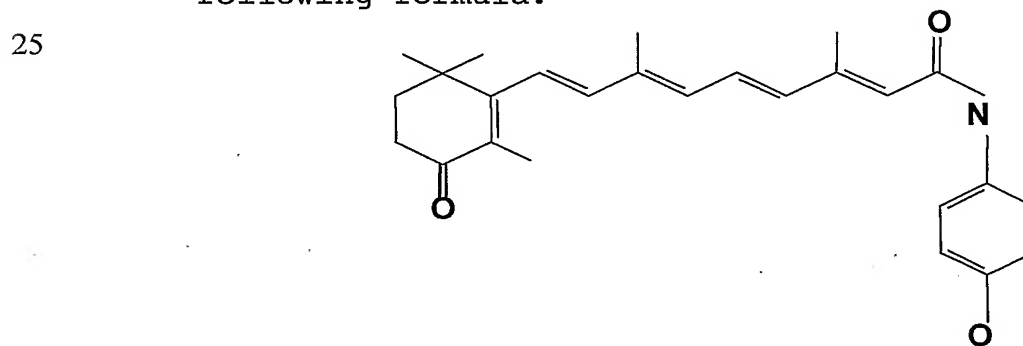
^aFemale Swiss nude mice were treated i.p. with 120 mg/kg of 4-oxo-4-HPR or 4-HPR for 4 days. Control mice were treated with the same solvent used for 4-oxo-4-HPR and 4-HPR. Three mice per group were used. Blood samples were collected at baseline and 5 h after the last treatment. The values are expressed as mean ±S.D.

CLAIMS

1. A drug consisting of a metabolite of fenretinide, or
N-(4-hydroxyphenyl)retinamide (4-HPR), specifically 4-
5 oxo-N-(4-hydroxyphenyl)retinamide (4-oxo-4-HPR) of the
following formula:



2. A drug according to claim 1, further comprising
fenretinide, or N-(4-hydroxyphenyl)retinamide (4-HPR).
3. A method of treating a hyperproliferative disorder in
a subject in need of such treatment, comprising
20 administering to said subject drug consisting of a
metabolite of fenretinide, or N-(4-
hydroxyphenyl)retinamide (4-HPR), specifically 4-oxo-
N-(4-hydroxyphenyl)retinamide (4-oxo-4-HPR) of the
following formula:



4. A method according to claim 3, comprising administering to said subject a combination, of 4-oxo-4-HPR and 4-HPR.
5. A method according to anyone of claims 3 and 4 in the treatment of ovarian carcinoma.
6. A method according to anyone of claims 3 and 4 in the treatment of breast tumor.
7. A method according to anyone of claims 3 and 4 in the treatment of neuroblastoma.
- 10 8. Use of a drug according to anyone of claims 1 and 2 in the treatment of ovarian carcinoma.
9. Use of a drug according to anyone of claims 1 and 2 in the treatment of breast tumor.
- 15 10. Use of a drug according to anyone of claims 1 and 2 in the treatment of neuroblastoma.

Fig. 1

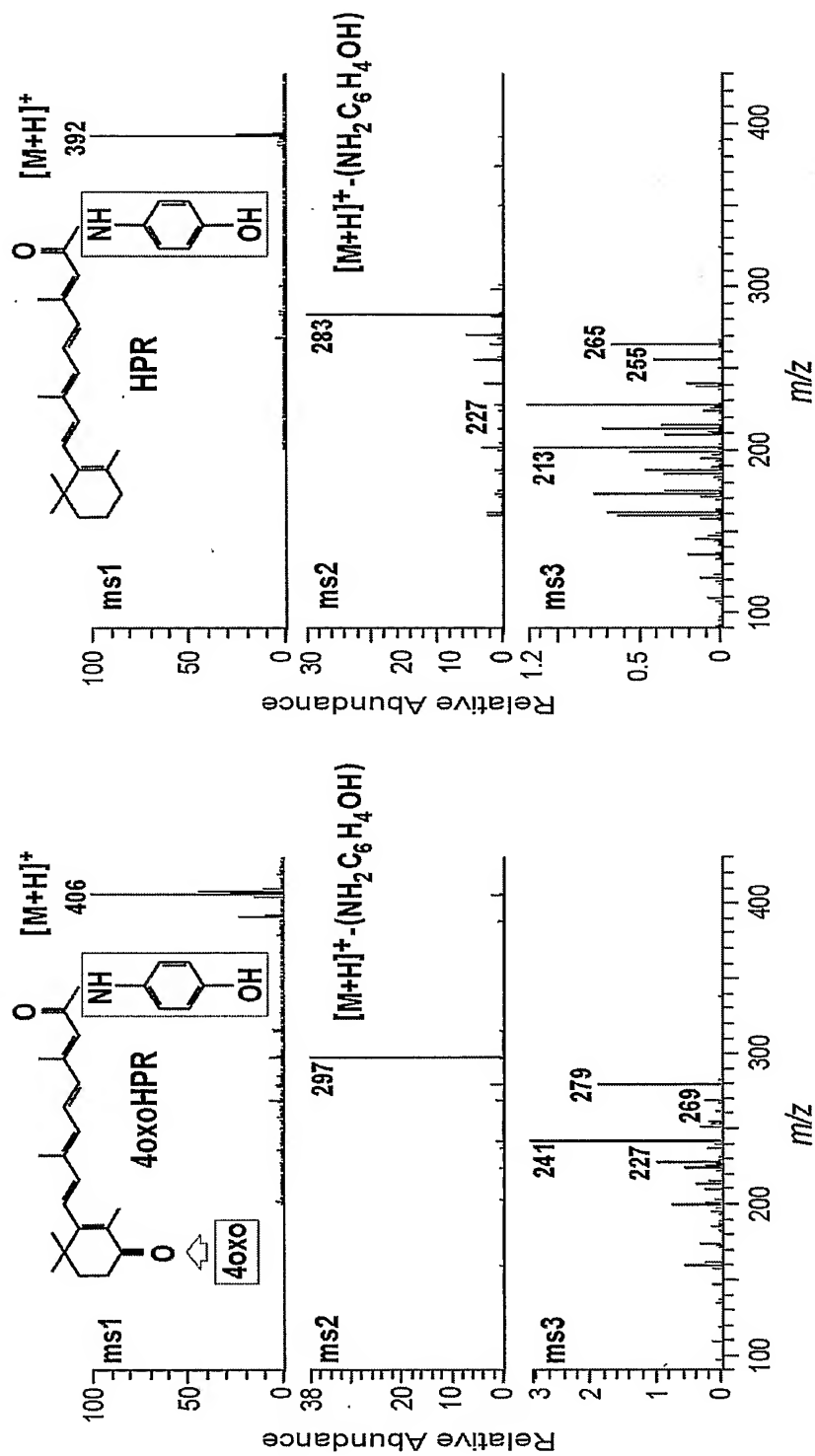


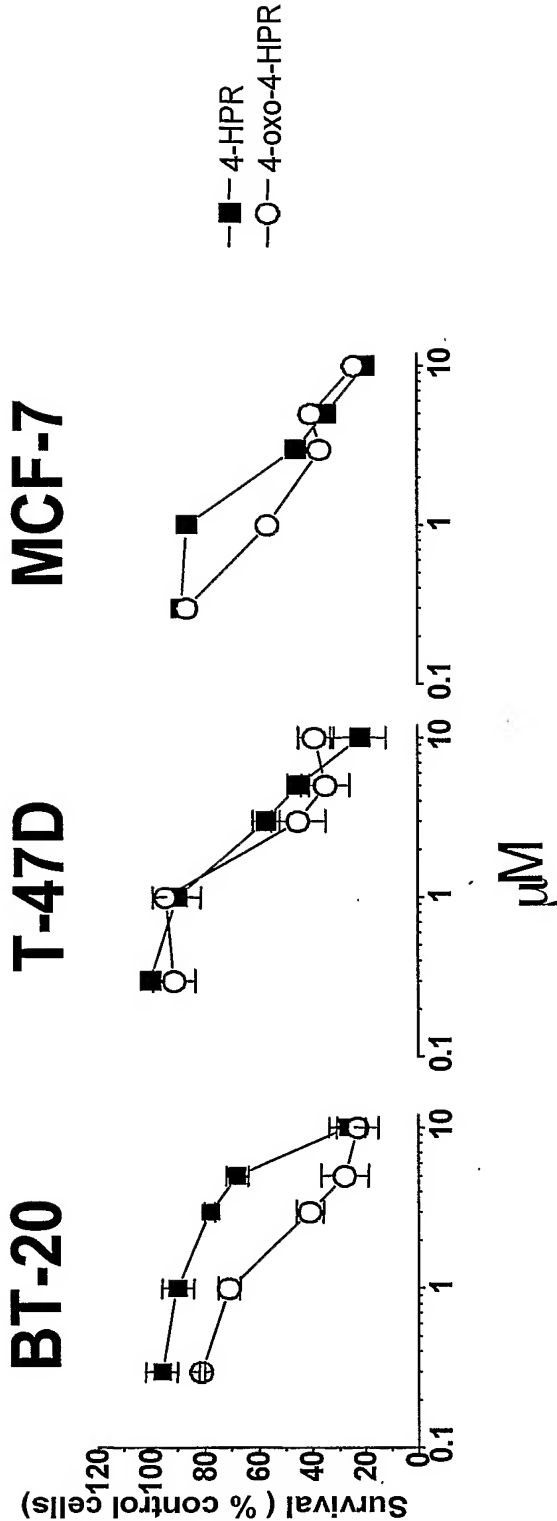
Figure 1 displays five dose-response curves showing the survival of different breast cancer cell lines (OVCA432, IGROV-1, OAW42, SKOV-3, and A2780/4-HPR) treated with 4-HPR (filled squares) and 4-oxo-4-HPR (open circles). The x-axis represents the concentration in μM on a logarithmic scale (0.1 to 10). The y-axis represents Survival (% control cells) from 0 to 120. Error bars represent standard deviation.

Legend:
 ■ 4-HPR
 ○ 4-oxo-4-HPR

Approximate Data Points:

Cell Line	Treatment	0.1 μM	1 μM	10 μM
OVCA432	4-HPR	~100	~85	~25
	4-oxo-4-HPR	~100	~85	~35
IGROV-1	4-HPR	~100	~85	~10
	4-oxo-4-HPR	~100	~85	~15
OAW42	4-HPR	~100	~85	~10
	4-oxo-4-HPR	~100	~85	~15
SKOV-3	4-HPR	~100	~85	~10
	4-oxo-4-HPR	~100	~85	~15
A2780/4-HPR	4-HPR	~100	~85	~10
	4-oxo-4-HPR	~100	~85	~15

Fig.3
Breast Tumor Cell Lines



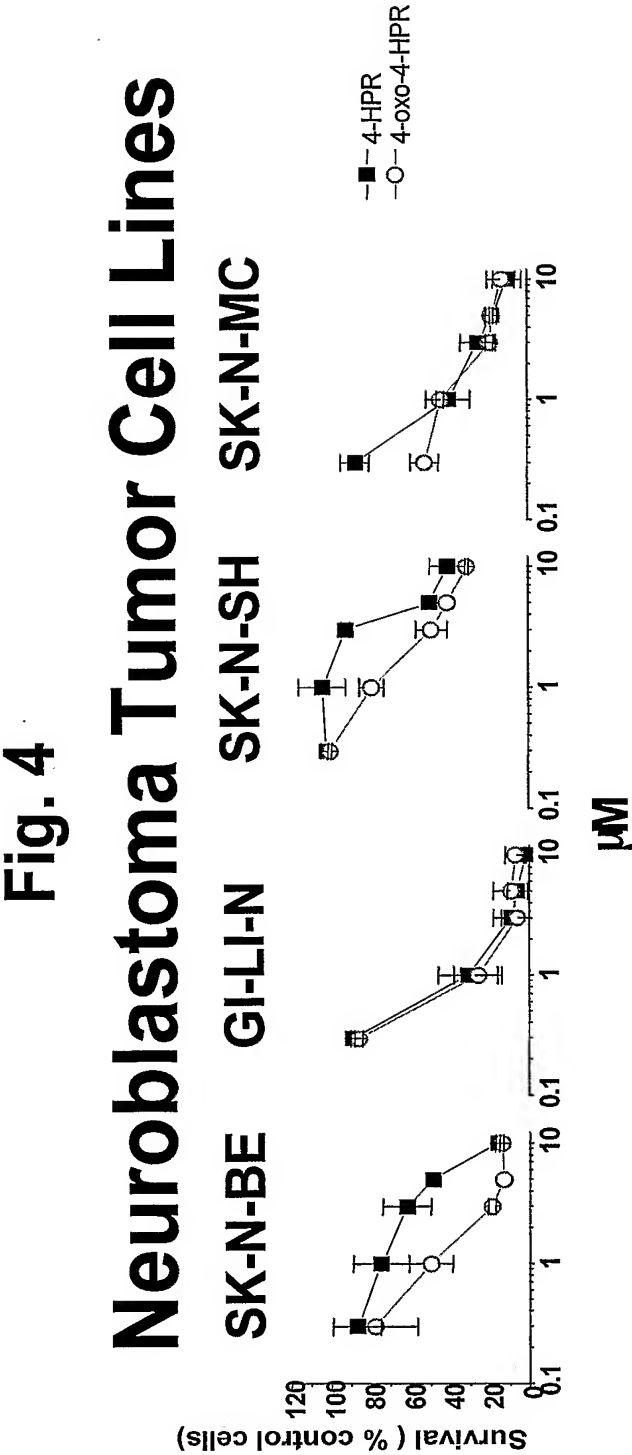


Fig. 5

APOPTOSIS ASSAY

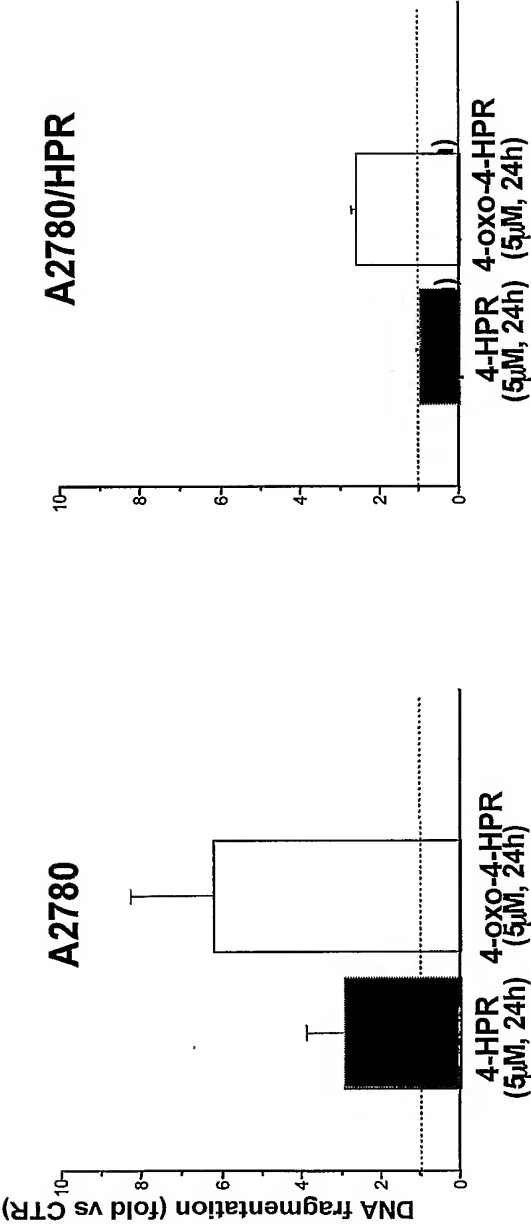
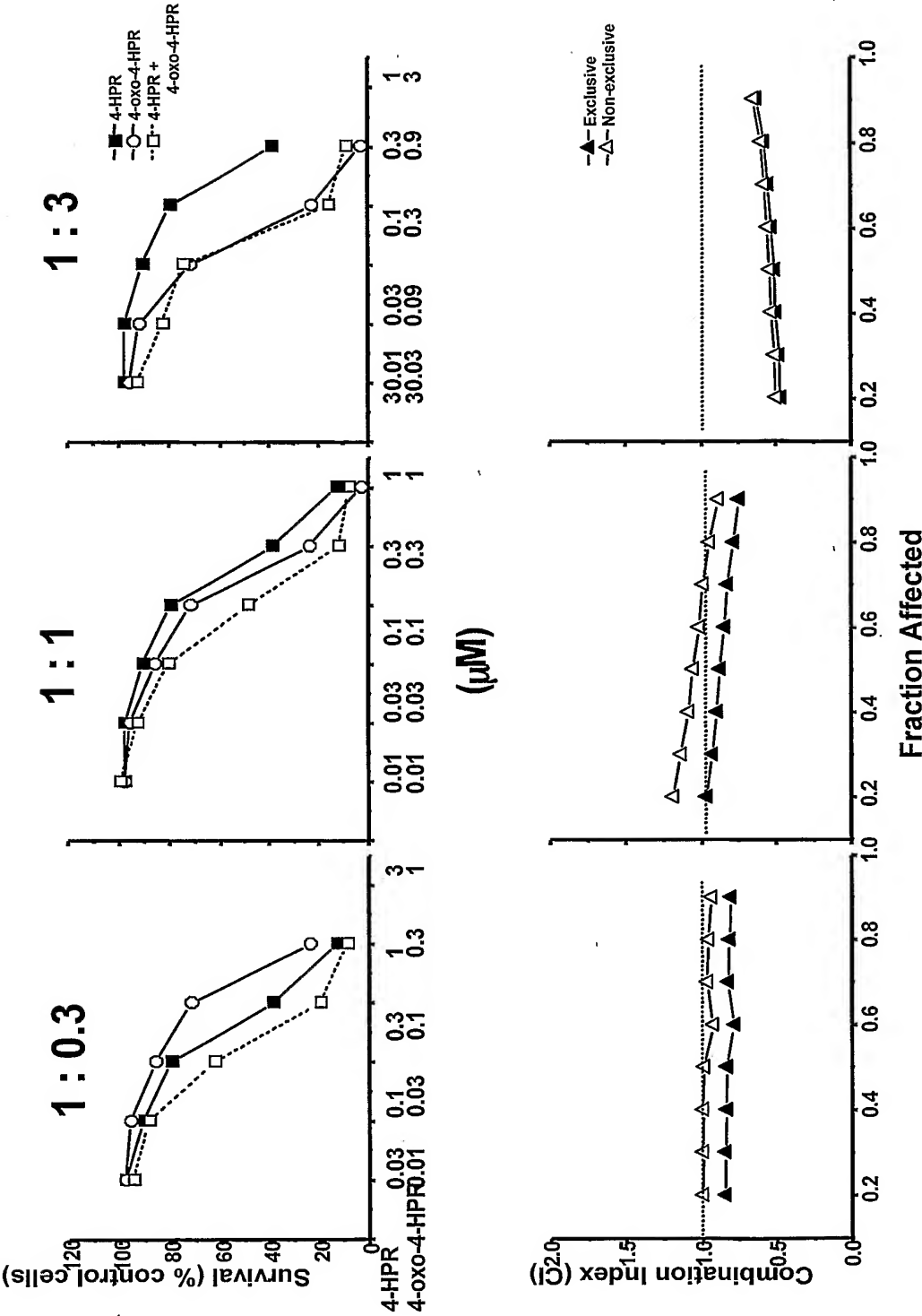


Fig. 6



INTERNATIONAL SEARCH REPORT

International Application No
T/IB2004/000789

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 A61K31/167 C07C233/00 A61P35/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C07C A61K A61P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, PAJ, BIOSIS, EMBASE, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>ULUKAYA E ET AL: "FENRETINIDE AND ITS RELATION TO CANCER" CANCER TREATMENT REVIEWS, SAUNDERS, US, vol. 25, no. 4, August 1999 (1999-08), pages 229-235, XP008009394 ISSN: 0305-7372 the whole document</p> <p style="text-align: center;">----- -/--</p>	1-10

☒ Further documents are listed in the continuation of box C.

☐ Patent family members are listed in annex.

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Date of the actual completion of the international search

3 November 2004

Date of mailing of the international search report

18/11/2004

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INTERNATIONAL SEARCH REPORT

International Application No

PCT/IB2004/000789

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>MEHTA R R ET AL: "Metabolism of N-(4-hydroxyphenyl)retinamide (4-HPR) to N-(4-methoxyphenyl)retinamide (4-MPR) may serve as a biomarker for its efficacy against human breast cancer and melanoma cells"</p> <p>EUROPEAN JOURNAL OF CANCER, vol. 34, no. 6, May 1998 (1998-05), pages 902-907, XP002303841 ISSN: 0959-8049 abstract</p>	1-10
A	<p>APPIERTO V ET AL: "Decrease in drug accumulation and in tumour aggressiveness marker expression in a fenretinide-induced resistant ovarian tumour cell line."</p> <p>BRITISH JOURNAL OF CANCER. 1 JUN 2001, vol. 84, no. 11, 1 June 2001 (2001-06-01), pages 1528-1534, XP002303842 ISSN: 0007-0920 abstract page 1528, left-hand column</p>	1-10
A	<p>OZOLS ROBERT F ET AL: "Specific keynote: Chemoprevention of ovarian cancer: The journey begins."</p> <p>GYNECOLOGIC ONCOLOGY, vol. 88, no. 1 Part 2, January 2003 (2003-01), pages S59-S66, XP002303843 ISSN: 0090-8258 page S62, last paragraph - page S63, paragraph 1</p>	1-10
A	<p>RIBATTI DOMENICO ET AL: "Fenretinide as an anti-angiogenic agent in neuroblastoma."</p> <p>CANCER LETTERS, vol. 197, no. 1-2, 18 July 2003 (2003-07-18), pages 181-184, XP002303844 ISSN: 0304-3835 abstract</p>	1-10